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Exhibit 2

An Immunomodulatory GpG Oligonucleotide for the Treatment of Autoimmunity via the Innate and Adaptive Immune Systems[1]

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Bacterial DNA and immunostimulatory CpG oligodeoxynucleotides (ODNs) activate the innate immune system to produce proinflammatory cytokines. Shown to be potent Th1-like adjuvants, stimulatory CpG motifs are currently used as effective therapeutic vaccines for various animal models of infectious diseases, tumors, allergies, and autoimmune diseases. In this study, we show that the application of an immunomodulatory GpG ODN, with a single base switch from CpG to GpG, can effectively inhibit the activation of Th1 T cells associated with autoimmune disease. Moreover, this immunomodulatory GpG ODN suppresses the severity of experimental autoimmune encephalomyelitis in mice, a prototypic Th1-mediated animal disease model for multiple sclerosis. *The Journal of Immunology,* **2003, 171: 4920–4926.**

Elements of both the adaptive and the innate immune system are transcribed and expressed at the site of disease in multiple sclerosis (MS)[3] (1–3). Components of the innate immune system are involved in several deleterious steps in the autoimmune cascade, including, but not limited to, the activation of complement and the development of membrane attack complexes in the CNS in MS (4). The innate immune system, however, provides a unique opportunity to suppress destructive autoimmunity. We demonstrate here an immunomodulatory DNA sequence that modulates the innate and/or the adaptive immune system and abrogates a prototypic model of autoimmune disease, experimental autoimmune encephalomyelitis (EAE).

It is well accepted in infectious diseases that the activation of innate immunity by specific immunostimulatory sequences in bacterial DNA requires a core unmethylated hexameric sequence motif consisting of 5′-purine-purine-cytosine-guanine-pyrimidine-pyrimidine-3′ (5). Bacterial DNA and synthetic oligodeoxynucleotides (ODN) containing this motif, referred to as "CpG" sequences, have the ability to stimulate B cells to proliferate and secrete IL-6, IL-10, and Ig (5, 6). CpG DNA also directly activates dendritic cells, macrophages, and monocytes to secrete Th1-like cytokines such as TNF-α, IL-6, and IL-12 and up-regulates the expression of MHC and costimulatory molecules (7–9). In mice, Toll-like receptor 9 (TLR-9) has been identified as the key receptor in the recognition of CpG motifs (10).

CpG DNA is recognized as a potent adjuvant for its ability to induce a strong Ab response and Th1-like T cell response to such Ags as hen egg lysozyme (11) and OVA (12). Currently, CpG DNA and CpG ODN are being used as therapeutic vaccines in various animal models of infectious diseases, tumors, allergic diseases, and autoimmune diseases (13). The success of CpG as a vaccine relies heavily on its effectiveness of inducing a strong Th1-like response and, in some instances, redirecting a Th2 response to a Th1 response, such as in the allergic asthma model (14, 15).

EAE is a Th1-mediated animal disease model of MS. Active induction of EAE requires immunization of the animal with myelin Ag or peptide in CFA, which contains heat-killed mycobacteria. Alternatively, it has been shown that CpG ODN is capable of completely substituting for heat-killed mycobacteria and priming encephalitogenic myelin-reactive T cells in vivo (16). Paradoxically, the insertion of three CpG motifs into the plasmid backbone of a DNA vaccine encoding MBP_{68-85} suppressed clinical signs of EAE in Lewis rats (17). Moreover, repeated injections of CpG ODN alone during the time course of EAE induction resulted in a milder disease incidence in Lewis rats (18). However, the use of a Th1 immunostimulatory product as treatment for a Th1-mediated disease may be detrimental in the long run, as has been demonstrated in clinical trials of MS using IFN-γ (19).

We report here that a single base substitution of a guanine for the cytosine in the stimulatory CpG ODN motif was effective in suppressing Th1 cell proliferation in vitro. Moreover, this immunomodulatory GpG motif appears to stimulate the proliferation of Th2 cells. We rationalized that such synthesized immunomodulatory sequences would be an attractive form of therapy in treatment of Th1-mediated autoimmune diseases such as EAE.

Materials and Methods

Mice

Female SJL/J and BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 5 wk of age. Mice were between 6 and 8 wk of age when experiments were initiated. TLR-9 knockout (KO) mice were a kind gift from Hemmi et al. (10) and bred in our own facilities. Myelin

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[3] Abbreviations used in this paper: MS, multiple sclerosis; ODN, oligodeoxynucleotide; TLR-9, Toll-like receptor 9; EAE, experimental autoimmune encephalomyelitis; WT, wild type; KO, knockout; MBP, myelin basic protein; IMO, immunomodulatory GpG ODN.

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basic protein (MBP) Ac_{1-11}-transgenic B10.PL female mice were a kind gift from the C. Garrison Fathman laboratory and bred in our own facilities.

Reagents

CpG ODN 5′-TGACTGTGAACGTTAGAGATGA-3′ and GpG ODN 5′-TGACTGTGAAGGTTAGAGATGA-3′ and a control CpC ODN 5′-CCCCCCCCCCCCCCCCCCCCCC-3′were all synthesized with a phosphorothioate backbone by Qiagen Operon (Alameda, CA). The underlining indicates the CpG motif and its corresponding single base substitution.

Proliferation assay

Whole splenocytes (1×10^6) were cultured in a 96-well microtiter plate and stimulated with different ODN in the indicated concentrations in triplicates for 72 h. Tissue culture medium for the assay consisted of RPMI 1640 supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (0.1 mg/ml), 2-ME (5×10^{-5} M), and 10% FCS. Wells were pulsed with 1 μCi [^{3}H]TdR (Amersham Pharmacia Biotech, Piscataway, NJ) for the final 16 h of culture and incorporated radioactivity was measured using a betaplate scintillation counter. In certain circumstances as indicated, 5×10^5 naive splenocytes were pulsed with indicated Ag and ODN for 24 h, then irradiated before the addition of 5×10^4 T cells for 72 h. Naive T cells from spleens and draining lymph nodes were isolated by a $CD3^+$ T cell column (R&D Systems, Minneapolis, MN) and determined to be at least 95% pure (data not shown). Committed $PLP_{139-151}$-reactive Th1 and Th2 T cells were obtained from our own clones (20). Wells were pulsed with 1 μCi [^{3}H]TdR for the final 16 h of culture and incorporated radioactivity was measured.

EAE induction

SJL female mice were immunized s.c. with 100 μg $PLP_{139-151}$ in PBS emulsified in CFA consisting of IFA (Sigma-Aldrich, St. Louis, MO) and 0.5 mg heat-inactivated *Mycobacterium tuberculosis* (strain H37 RA; Difco, Detroit, MI). At the same time, mice were injected i.p. with 50 μg ODN in 200 μl of PBS. Animals were clinically scored daily. Grade 1, tail paralysis; grade 2, hind limb paraparesis; grade 3, hind limb paralysis; grade 4, complete paralysis (tetraplegy); and grade 5, death.

Quantitative real-time PCR

Whole splenocytes (1×10^7) from naive SJL mice were cultured for 72 h in enriched RPMI 1640 and 10% FCS with the indicated ODN at a concentration of 5 μg/ml. Total RNA was isolated using RNeasy (Qiagen, Valencia, CA) and converted to cDNA using SuperScript II RNase H^- Reverse Transcriptase (Invitrogen, Carlsbad, CA) for first-strand cDNA synthesis. The cDNA product was used for real-time quantitative PCR using a high-speed thermal cycler (LightCycler3; Roche Diagnostics, Indianapolis, IN) and detection of product by SYBR Green I (Qiagen). PCR primers for detection of I-A_β^s consisted of a sense primer, 5′-AGGCATT TCGTGTTCCAGTT-3′, and antisense primer, 5′-GTCTCCACCCCCTC GTAGTT-3′. The amplification cycle was: 95°C for 900 s, 60 cycles of 94°C for 15 s, 56°C for 20 s, 72°C for 15 s; 65°C for 15 s, and 40°C for 30 s. Melting curves confirmed that only one product was amplified. Specific cDNA was quantified with a standard curve based on known amounts of amplified β-actin fragment.

FACS analysis

Whole splenocytes (1×10^7) from naive SJL mice were cultured for 72 h in enriched RPMI 1640 and 10% FCS with the indicated ODN at a concentration of 5 μg/ml. Cells were harvested and analyzed on a FACScan flow cytometer (BD Biosciences, Mountain View, CA) using CellQuest software (BD Immunocytometry Systems). The following Ab conjugates were used: FITC anti-mouse CD40, clone HM40-3, FITC anti-mouse CD80, clone 16-10A1; FITC anti-mouse CD86, clone GL1; FITC anti-mouse I-A^k, clone 10-3.6, and FITC anti-mouse CD1.1, clone 1B1. All Abs were purchased from BD PharMingen (San Diego, CA).

Western immunoblotting analysis

Whole splenocytes (1×10^7) from naive SJL mice were cultured for 72 h in enriched RPMI 1640 and 10% FCS with the indicated ODN at a concentration of 5 μg/ml. Cells were harvested and protein was extracted using M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Protein concentrations were determined using a BCA Protein Assay kit (Pierce). A total of 20 μg of each protein sample per lane was resolved on a 4–15% Tris-HCl Ready Gel (Bio-Rad, Hercules, CA) and transferred onto Hybond-P polyvinylidene difluoride membranes. After blocking of membranes with 5% nonfat dry milk, the blots were probed with phospho-IκB-α (Ser^{32}) Ab (Cell Signaling Technology, Beverly, MA) and visualized with HRP-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech) and an ECL+Plus detection system (Amersham Pharmacia Biotech).

Cytokine analysis

Whole splenocytes (1×10^7) from naive SJL mice were cultured for 72 h in enriched RPMI 1640 and 10% FCS with the indicated ODN at the indicated microgram per milliliter concentrations. Cells were harvested and the supernatants were collected and tested by sandwich ELISA using standard ELISA kits (BD PharMingen).

Results

GpG ODN suppresses CpG ODN stimulation of naive splenocytes in vitro

Stimulatory CpG ODN is known to activate immune cells derived from spleens including dendritic cells, macrophages, T cells, and B cells (5–9). We first investigated the effects of immunomodulatory GpG ODN (IMO) on the stimulatory CpG ODN by measuring overall proliferation of naive splenocytes. We constructed a 22-mer ODN sequence containing a single 5′-AACGTT-3′ (CpG ODN) or 5′-AAGGTT-3′ (GpG ODN) sequence with a phosphorothioate backbone to protect the DNA from nuclease degradation (8). To determine whether the addition of IMO would counteract the effects of stimulatory CpG ODN, isolated naive whole splenocytes were cultured with 5 μg/ml stimulatory CpG ODN alone and with increasing concentrations of IMO. After 48 h, whole splenocyte proliferation decreased 2-fold upon the addition of 1 μg/ml GpG ODN and decreased 3-fold with the addition of 5 and 10 μg/ml GpG ODN (Fig. 1*A*).

TLR-9 has been shown to recognize bacterial DNA CpG motifs (10). We investigated whether a simple C to G base pair switch would alter this recognition. To this end, isolated naive whole splenocytes from TLR-9 wild-type (WT) and TLR-9 KO mice were separately cultured with 5 μg/ml CpG ODN, GpG ODN, a control ODN, and combinations of both. As a separate control, 1 μg/ml LPS was added to show that the TLR-9 KO splenocytes were still capable of proliferating to a nonspecific mitogenic stimulus. The addition of stimulatory CpG ODN resulted in a strong proliferative response that was significantly suppressed by the addition of immunomodulatory GpG ODN and less by the control ODN (Fig. 1*B*). The combination of LPS with stimulatory CpG ODN increased splenocyte proliferation as compared with LPS stimulation alone. However, the immunomodulatory effects of the GpG ODN were still evident even with the addition of LPS.

The absence of TLR-9 receptor from the TLR-9 KO splenocytes abrogated the proliferative effects of CpG ODN as compared with TLR-9 WT splenocytes. Similarly, the TLR-9 KO splenocytes did not respond to the IMO. Upon the addition of LPS to the TLR-9 KO splenocytes, we found that the IMO alone or in combination with stimulatory CpG ODN did not affect the proliferative response compared with TLR-9 WT splenocytes. This implies that IMO may be preventing stimulatory CpG ODN from proceeding through the TLR-9 signaling pathway.

GpG ODN inhibits phosphorylation of IκB-α at Ser^{32}

The recognition of stimulatory CpG ODN by TLR-9 triggers the induction of cell signaling pathways culminating in NF-κB activation (13). To understand the mechanism of IMO on stimulatory CpG ODN, we investigated the role of NF-κB activity through the phosphorylation of IκB-α at Ser^{32}. Western blot analysis of extracts from splenocytes activated with the indicated ODN confirms the phosphorylation of IκB-α at Ser^{32} by stimulatory CpG ODN (Fig. 2, *lane 2*). Accordingly, IMO did not induce phosphorylation of IκB-α at Ser^{32} (Fig. 2, *lane 3*). Interestingly, the combination of stimulatory CpG ODN and IMO resulted in a marked reduction in phosphorylation of IκB-α at Ser^{32} (Fig. 2, *lane 5*).

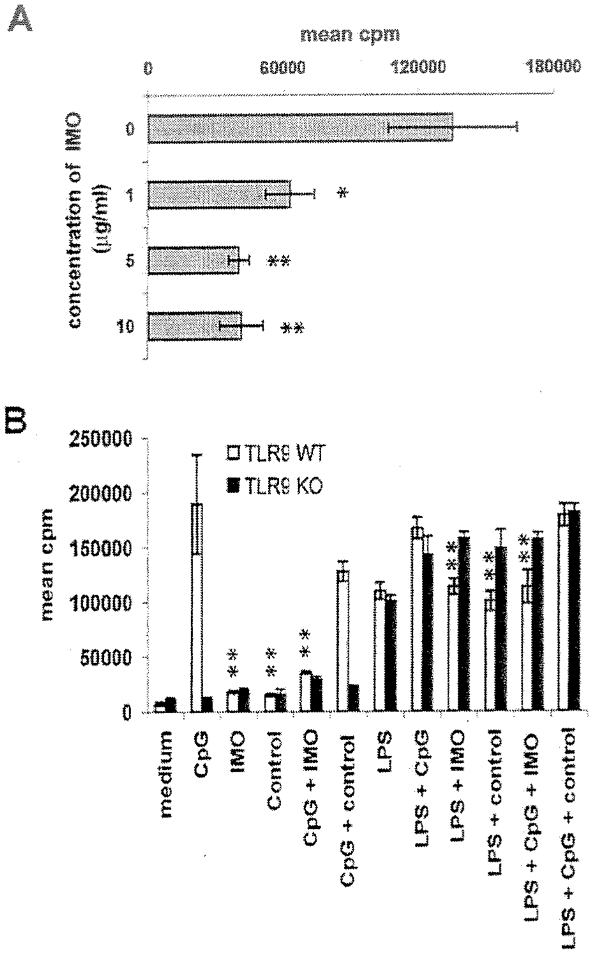

FIGURE 1. IMO suppresses whole splenocyte proliferation mediated by stimulatory CpG ODN and is dependent on TLR-9. *A*, Whole splenocytes were cultured for 72 h with stimulatory CpG ODN and increasing concentrations of IMO ODN as indicated. Wells were pulsed with [^{3}H]TdR for the final 16 h of culture before incorporated radioactivity was measured. Each data point represents the mean of triplicate wells ± SD. *B*, Whole splenocytes from TLR-9 WT and TLR-9 KO mice were isolated and cultured with stimulatory CpG ODN, IMO, control ODN, or LPS for 72 h. Wells were pulsed with [^{3}H]TdR for the final 16 h of culture before incorporated radioactivity was measured. Each data point represents the mean of triplicate wells ± SD. This figure is a representative of two to three independent experiments. The symbols indicate results significantly different from the CpG ODN-stimulated condition and the LPS with CpG ODN-stimulated condition, respectively (*, $p < 0.05$; **, $p < 0.01$) as determined by the ANOVA test.

GpG ODN reduces MHC class II expression

CpG ODN have been shown to increase MHC class II expression (8). To investigate MHC class II expression, naive splenocytes were removed from animals and incubated for 72 h with either stimulatory CpG ODN or the IMO. RNA was purified from harvested cells and cDNA was synthesized with an oligo(dT) primer. Quantitative PCR analysis was performed to quantitate the relative concentration of message for the MHC class II molecule. Signals were normalized relative to the quantity of message for β-actin. MHC class II mRNA was increased in stimulatory CpG ODN-incubated splenocytes but not in IMO-incubated splenocytes (Fig. 3*A*).

To further confirm the down-regulation of MHC class II expression by IMO, we examined cell surface expression of MHC class II protein by FACScan analysis. As above, naive splenocytes were removed from animals and incubated for 72 h with the indicated concentrations of IMO, stimulatory CpG ODN, or irrelevant control ODN. The IMO suppressed the activation of MHC class II cell surface expression by the stimulatory CpG ODN in a dose-dependent manner (Fig. 3*B*).

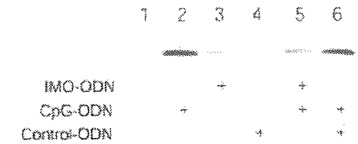

FIGURE 2. IMO inhibits phosphorylation of IκB-α at Ser^{32}. Naive splenocytes were cultured in the presence of the indicated ODN. The phosphorylation of IκB-α at Ser^{32} was determined by Western blot analysis of each protein extract. IκB-α is activated in the presence of stimulatory CpG (*lane 2*) or stimulatory CpG ODN and control ODN (*lane 6*), but becomes reduced in activation with the addition of the IMO to the stimulatory CpG ODN (*lane 5*). Similar data were obtained in a repeat experiment.

GpG ODN reduces APC activation, but increases CD1d expression

To determine whether IMO reduced APC activation, cell surface expression of various APC activation markers was also analyzed. Naive splenocytes were incubated for 72 h with the indicated concentrations of GpG, stimulatory, or irrelevant control ODN. Cells were harvested and measured by FACScan analysis as described in

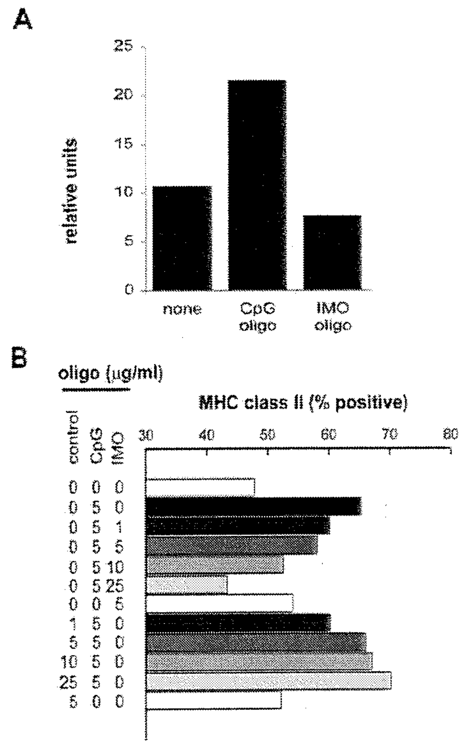

FIGURE 3. IMO reduces MHC class II expression. *A*, Naive splenocytes were cultured in the absence or presence of either stimulatory CpG ODN or IMO. cDNA was synthesized from purified RNA for quantitative PCR analysis. The quantity of RNA for MHC class II is indicated as the relative units compared with quantity of β-actin present in each sample. *B*, Naive splenocytes were cultured in the presence of the indicated amount of each ODN. The percentage of cells positive for MHC class II expression was analyzed by FACS, and as shown there is a dose-dependent inhibition of the expression of MHC class II with increasing concentrations of the IMO. Similar data were obtained in a repeat experiment.

Materials and Methods. The IMO suppressed the cell surface expression of CD40 (Fig. 4*A*), CD80 (Fig. 4*B*), and CD86 (Fig. 4*C*) in a dose-dependent manner. In contrast, the expression of the glycolipid presentation molecule, CD1d (Fig. 4*D*), was increased by the IMO in a dose-dependent manner.

GpG ODN suppresses Th1 cytokine production

To profile the effect on cytokine production of immune cells by the inhibitor ODN, naive splenocytes were removed from animals and incubated for 72 h with the indicated concentrations of IMO, stimulatory CpG ODN, or irrelevant control ODN. The IMO alone suppressed the production of IL-6 (Fig. 5*A*) and IL-12p40 (Fig. 5*B*), and when combined with stimulatory CpG ODN suppressed cytokine production in a dose-dependent manner.

GpG ODN suppresses CpG ODN stimulation of naive MBP Ac_{1-11}-transgenic T cells

Having shown that IMO can down-regulate specific activation markers and MHC class II expression on APCs, we next determined whether the IMO could alter the Ag presentation and stimulatory properties of APCs before encountering uncommitted T cells specific for a myelin self-Ag. Isolated naive B10.PL whole splenocytes were initially cocultured with 5 μg/ml MBP Ac_{1-11} and 5 μg/ml of the indicated ODN for 24 h before being irradiated. Naive MBP Ac_{1-11} recognizing T cells were isolated from spleen and draining lymph nodes of MBP Ac_{1-11} TCR-transgenic mice and purified by a CD3$^+$ T cell isolation column before being added to stimulated irradiated splenocytes. Preincubation of APCs with the stimulatory CpG ODN and MBP Ac_{1-11} peptide was capable of stimulating naive MBP Ac_{1-11} TCR-transgenic T cells 2-fold more than with Ag alone (Fig. 6*A*). In comparison, preincubation of APCs with IMO did not augment the stimulation by peptide alone. Most importantly, preincubation of APCs with a combination of both GpG ODN and CpG ODN resulted in a decrease in the proliferation of TCR-transgenic T cells as compared with CpG ODN alone.

GpG ODN suppresses Th1 cell but enhances Th2 cell proliferation

Having demonstrated that IMO was capable of suppressing naive uncommitted myelin-specific T cells, we hypothesized that it might also have differential effects on committed Th1 or Th2 cells. To this end, naive whole SJL splenocytes used as APCs were preincubated with 10 μg/ml $PLP_{139-151}$ and 5 μg/ml of the indicated ODN for 24 h before being irradiated. A $PLP_{139-151}$-specific Th1 cell line was then added to the culture and the proliferation of these T cells was measured. Stimulatory CpG ODN increased the proliferation of the Th1 cell line, whereas the IMO suppressed its proliferation (Fig. 6*B*). The combination of IMO and stimulatory CpG ODN was capable of somewhat decreasing the augmentation of Th1 cell proliferation caused by CpG ODN alone.

Similar investigations were undertaken with a $PLP_{139-151}$-specific Th2 cell line. Naive whole SJL splenocytes were preincubated with 10 μg/ml $PLP_{139-151}$ and 5 μg/ml of the indicated ODN for 24 h before being irradiated. A $PLP_{139-151}$-specific Th2 cell line was

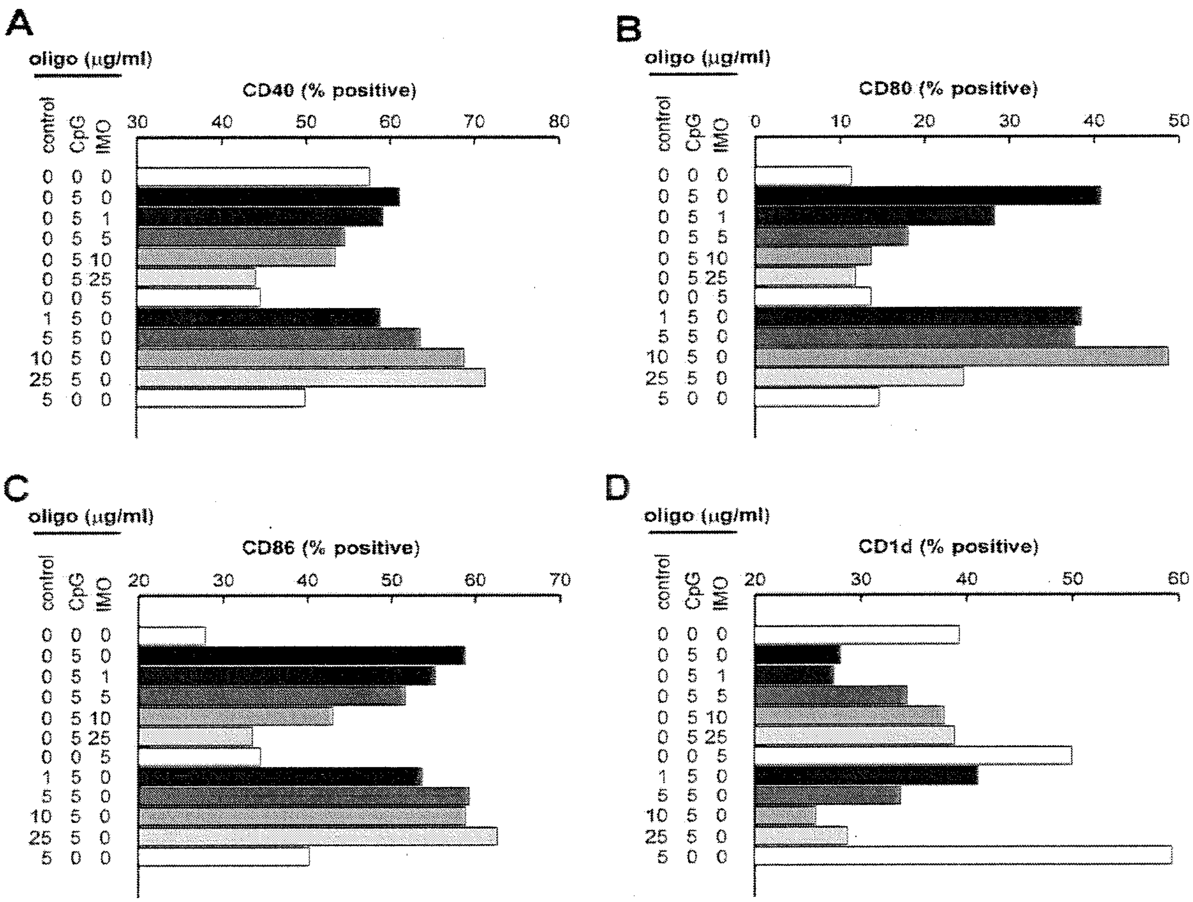

FIGURE 4. Effect of IMO on expression of APC activation markers. Naive splenocytes were cultured with the indicated concentrations of stimulatory CpG ODN, IMO, or control ODN. FACScan analysis was used to assess expression of CD40 (*A*), CD80 (*B*), CD86 (*C*), and CD1d (*D*). There is a dose-dependent reduction in expression of CD40, CD80, and CD86, but a dose-dependent increase in expression of CD1d with the IMO. Similar data were obtained in a repeat experiment.

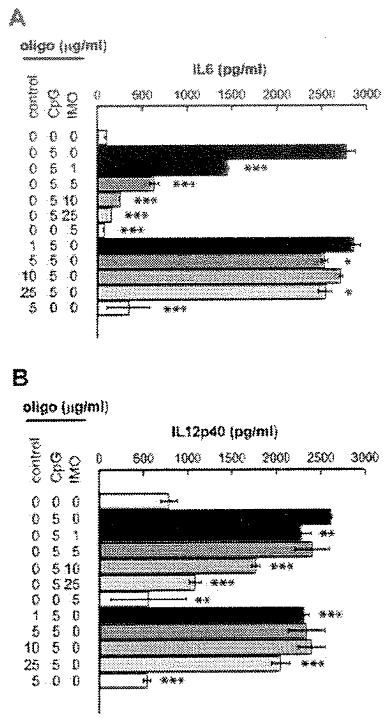

FIGURE 5. IMO suppresses Th1 cytokine production. Naive splenocytes were cultured with the indicated concentrations of stimulatory CpG ODN, IMO, or control ODN. IL-6 (*A*) and IL-12p40 (*B*) production were measured by ELISA. As indicated, there is a dose-dependent inhibition of the production of both IL-6 and IL-12p40 with increasing concentrations of the IMO. Each data point represents the mean of triplicate wells. Similar data were obtained in a repeat experiment. The symbols indicate results significantly different from the CpG ODN-stimulated condition (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) as determined by the ANOVA test.

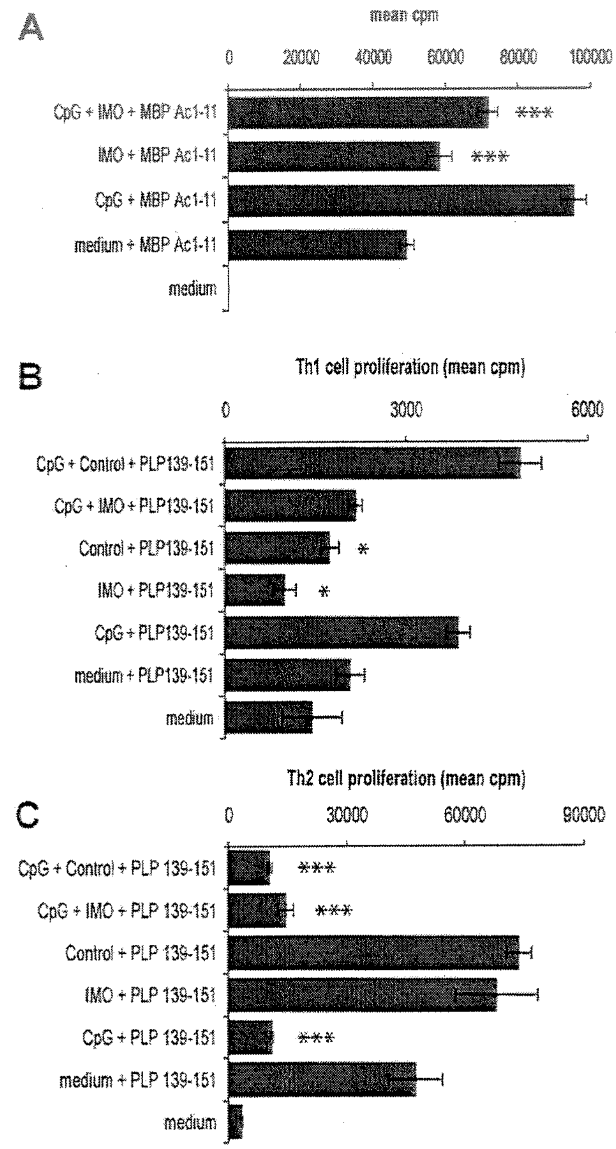

FIGURE 6. Differential effects of the IMO on various purified T cells. *A*, IMO suppresses CpG ODN stimulation of naive uncommitted MBP Ac_{1-11} TCR-transgenic T cells. As indicated in the *first row*, incubation with IMO suppressed the augmented proliferation induced by stimulatory CpG ODN. *B*, IMO suppresses a $PLP_{139-151}$-specific Th1 cell line. IMO does not stimulate Th1 cell proliferation and in fact decreases the proliferation induced by CpG ODN slightly. *C*, In contrast, IMO does not inhibit the proliferation of a $PLP_{139-151}$-specific Th2 cell line. Note that the CpG ODN reduces the proliferation of this Th2 cell line. Each data point represents the mean of triplicate wells ± SD. Similar data were obtained in repeat experiments. The symbols indicate results significantly different from the CpG ODN-stimulated condition (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) as determined by the ANOVA test.

then added and the proliferation of these T cells was measured. Stimulatory CpG ODN suppressed the proliferation of the Th2 cell line (Fig. 6*C*). Surprisingly, the IMO enhanced the proliferation of the Th2 cell line as did the control ODN. However the combination of IMO and CpG ODN caused a decrease in the enhancement of proliferation. Thus, the CpG ODN acts as an inhibitor of Th2 cells, but the IMO and control ODN stimulate Th2 cells.

GpG ODN suppresses $PLP_{139-151}$-mediated EAE

These results led us to explore the use of IMO as a method of prevention of EAE induction. SJL/J mice were immunized s.c. for disease induction with 100 µg of $PLP_{139-151}$ peptide in CFA. On the day of immunization, 50 µg of the indicated ODN resuspended in 200 µl of PBS was also administered i.p. as a single injection. For the combination of CpG ODN plus IMO and CpG ODN plus control ODN, 50 µg of each ODN was administered. Mice treated with just a single injection of IMO exhibited an overall decreased disease severity as compared with mice treated with PBS, control ODN, or stimulatory CpG ODN (Fig. 7*A*). The combination of CpG ODN plus IMO was more effective in preventing disease than the combination of CpG ODN plus control ODN.

Thirty-seven days after $PLP_{139-151}$ immunization and ODN treatment, draining lymph nodes were removed from the animals. Whole lymph node cells were restimulated in vitro for assessment of Ag-specific proliferation. An irrelevant control peptide and a nonspecific activator, Con A, were used as negative and positive controls, respectively. T cells from mice treated with IMO and control ODN had reduced proliferation to $PLP_{139-151}$ compared with T cells from mice treated with stimulatory CpG ODN (Fig. 7*B*). Interestingly, T cells from mice treated with the combination of CpG ODN plus IMO were significantly reduced in Ag-specific proliferation compared with all other treatment groups.

Given that a single injection of the IMO alone was able to significantly reduce disease severity as a preventive form of therapy, we hypothesized that treatment of disease beginning at the peak of onset with IMO would also reduce disease severity. SJL/J mice were immunized s.c. for disease induction with 100 µg of $PLP_{139-151}$ peptide in CFA. On day 14 following immunization, at the peak of acute EAE disease, 50 µg of the IMO resuspended in 200 µl of PBS was

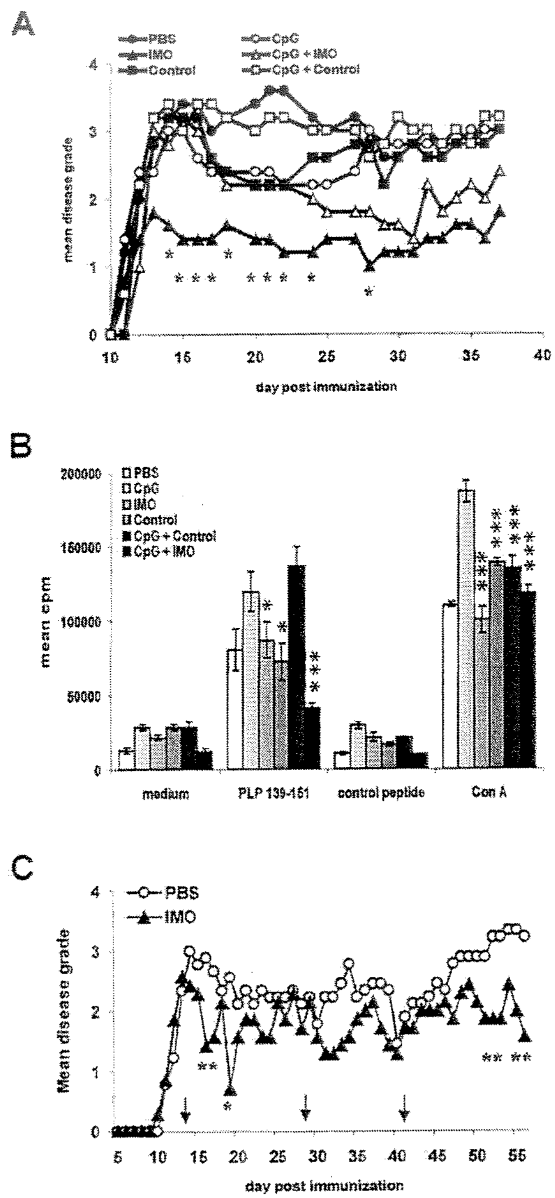

FIGURE 7. IMO suppresses $PLP_{139-151}$-mediated EAE. *A*, SJL/J mice were induced with EAE and treated with ODN at the same time. Shown is a representative experiment of three independent experiments with similar results. Asterisks (*) indicate the days in which the mean disease score of the IMO group was statistically different ($p < 0.05$) from the PBS control group as determined by the ANOVA test. *B*, Thirty-seven days after $PLP_{139-151}$ immunization and ODN treatment, draining lymph nodes from each group were removed. Whole lymph node cells were restimulated in vitro for assessment of Ag-specific proliferation. Each data point represents the mean of triplicate wells ± SD. The symbols indicate results significantly different from the CpG ODN-treated group (*, $p < 0.05$; ***, $p < 0.001$) as determined by the ANOVA test. *C*, SJL/J mice were immunized s.c. with $PLP_{139-151}$ peptide in CFA. IMO was administered i.p. on days 14, 28, and 42 (indicated with arrows) after the peptide immunization. Asterisks (*) indicate the days in which the mean disease score of the IMO group was statistically different ($p < 0.05$) from the untreated control group as determined by the ANOVA test.

administered i.p. This treatment was repeated on days 28 and 42 following disease induction. Mice treated with IMO on average showed decreased severity as compared with PBS-treated control mice (Fig. 7*C*).

Although IMO treatment of EAE was not as dramatic as prevention of EAE, there are apparent indications that after each injection of IMO, overall disease severity is reduced for several days. Therefore, future treatment experiments involving an increase in IMO administration from biweekly to weekly injections and an increase in ODN dosage may very well lead to a more significant reduction in the disease severity of EAE.

Discussion

In the current study, we show that modulation of the innate immune system is a viable method of treating autoimmune disease. We demonstrate that a single base pair change from C to G can drastically alter a strong stimulatory reaction by a CpG motif to an immunomodulatory reaction by a GpG motif. The GpG motif may be recognized by the same TLR-9 signaling pathway as the stimulatory CpG motif. The phosphorylation of $I\kappa B$-α at Ser^{32} is essential for the release of active NF-κB, which has been reported as a component of the cell signaling pathway activated by TLR-9 binding of the stimulatory CpG motif. We show the reduction of phosphorylation of $I\kappa B$-α at Ser^{32} in the presence of IMO alone or in combination with stimulatory CpG ODN.

Whereas the stimulatory CpG ODN up-regulates MHC class II expression, APC cell surface activation markers, and Th1 cytokine secretion, we show that the IMO can effectively suppress all of these same responses. We postulated that part of the mechanism of action of IMO consists of effects on Ag presentation. We confirmed this by preincubating APCs with ODN and peptide and assessing the influence of this preincubation on T cells. IMO abolished the enhancement of T cell proliferation caused by stimulatory CpG ODN. Moreover, this effect appears to differentially affect T cell subtypes, as IMO alone appears to enhance the proliferation of Th2 cells but not Th1 cells. The most promising observation for the immunomodulatory effectiveness of the GpG ODN was our in vivo data. A single injection of the IMO was sufficient in reducing the overall average disease grade for the prevention of EAE. Moreover, initial studies in the treatment of EAE indicate that repeated injections of IMO will also reduce the overall average disease grade of EAE.

Although in nearly every instance the IMO suppressed the activation of APCs or T cells, including Th1 T cells, there were two instances in which it did not. IMO increased expression of CD1d on APCs, and IMO did not suppress the proliferation of Th2 T cells whereas stimulatory CpG ODN did. These two results suggest that part of the mechanism involved in the efficacy of IMO in preventing EAE is the activation of CD1d on APCs and the maintenance of proliferation of protective Th2 cells while suppressing encephalitogenic Th1 cells. Perhaps the activation of CD1d is involved in the stimulation of protective or suppressive NK T cells in Th1-mediated autoimmune diseases.

In addition to the use of immunomodulatory ODN alone, they could also be used to improve other methods of therapy. We and others have reported the effectiveness of DNA therapy with bacterial vectors for treatment of EAE (20–22) and diabetes (23, 24). However, these same bacterial vectors contain CpG motifs that could be acting as adjuvants for uncontrolled stimulation. Therefore, combining DNA therapy with IMO could potentially enhance the effectiveness of DNA vaccines by reducing the effect of nonspecific stimulation.

We have shown here evidence that the innate immune pathway could be used as an alternative route to treat autoimmune disease. Innate immune activation provides signals that are necessary for lymphocyte activation or differentiation (25). This in turn influences the induction of an appropriate adaptive immune response (26). In the case of an autoimmune disease, the final destructive

outcome of the adaptive immune response to self-Ags is initiated by "danger" signals from the innate immune response to self-Ag (26–28). Therefore, to treat autoimmune disease, it may be necessary to also target the innate part of the immune system.

The modulation of autoimmune disease through the regulation of the innate immune system has been previously addressed by others. Vaccination with heat shock protein 60 has been reported to inhibit disease in animal models of insulin-dependent diabetes mellitus (29, 30) and adjuvant arthritis (31). Activation of NK T cells by α-galactosylceramide has been shown to prevent disease in animal models of insulin-dependent diabetes mellitus (32–34) and MS (35, 36). Inhibition of complement by the administration of anti-C5 Ab (37) or the delivery of complement receptor 1 (38, 39) prevented the progression of collagen-induced arthritis in rodents.

In summary, the current data suggest that there are two possible mechanisms of action whereby IMO alter the immune system in the context of autoimmune disease. One may be through the TLR-9 signaling pathway and consequent APC activation. Alternatively, several pieces of data indicate that IMO may be acting to alter the immune system through a unique pathway as suggested by the in vitro results demonstrating the activation of CD1d expression and the maintenance of proliferation of protective Th2 T cells.

The use of IMO to treat EAE appears to be a novel method of suppressing the destructive cascade caused by the immune response to self-Ags in EAE. Therefore, IMO alone or in combination with Ag-specific treatments may prove to be a powerful method of controlling autoimmune diseases such as MS, juvenile diabetes, and rheumatoid arthritis.

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